

# Mistletoe lectin dissociates into catalytic and binding subunits before translocation across the membrane to the cytoplasm

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**Abstract** Hybridomas producing monoclonal antibodies (mAbs) against the mistletoe lectin A-chain (MLA) were obtained to investigate the intracellular routing and translocation of ribosome-inactivating proteins. Anti-MLA mAb MNA5 did not bind the holotoxin but interacted with isolated MLA. This epitope was not recognized upon MLA denaturation or conjugation of MLA with the ricin binding subunit (RTB). Furthermore, the mAbs did not appreciably react with a panel of MLA synthetic octapeptides linked to the surface of polyethylene pins. A study of the cytotoxicity of mistletoe lectin, ricin, and chimeric toxin MLA/RTB for the hybridomas revealed that interchain disulfide bond reduction and subunit dissociation are required for cytotoxic activity of mistletoe lectin.

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**Key words:** Mistletoe lectin; Ribosome-inactivating protein; Monoclonal antibody; Membrane translocation

## 1. Introduction

Mistletoe lectin from *Viscum album* is a type II ribosome-inactivating protein (RIP), comprising a toxic A-chain and a carbohydrate-binding B-chain linked by a disulfide bond, like other members of this family including ricin, abrin, and mod-ecin [1]. These toxic proteins are potent inhibitors of eukaryotic protein synthesis at the ribosomal level. The A-chain inhibits protein synthesis by enzymatic depurination of an adenosine residue in a highly conserved loop of the 28S ribosomal RNA [2]. Since type II RIP are very efficient in target cell toxicity, they have been used in producing immunotoxins, pharmaceuticals against tumor and immunocompetent cells to be applied in autoimmune diseases and transplantology [3].

Endocytosis of the protein is mediated by the lectin B-chain which has multiple potential sugar binding sites, interacting with glycosidic residues on the surface of target cells. Following endocytosis, the process of intracellular trafficking is not

clear, but recent work implicates the endoplasmic reticulum (ER) in the toxin translocation [4]. The efficacy of RIP II in inhibiting mammalian protein synthesis is dependent on the toxin translation efficacy. In the case of ricin, the B-chain is believed to mediate the translocation of the A-chain [5]. Unlike the bacterial toxins, no distinct translocation domain has been identified in RIP II. Understanding the mechanism by which RIP II reaches the cytosol would help in designing better toxin and immunotoxin molecules with new structural motifs for improved translocation.

It is not yet clear whether the toxins are translocated as holomolecules or are reduced and then translocated. There is evidence for translocation of the holotoxin: (1) a ricin with a non-dissociating bond between A- and B-chains has the same cytotoxic activity as the native protein [6]; (2) most RIP II holomolecules have enzymatic activity sufficient for inhibition of protein synthesis [2]; (3) the depth of ricin permeation into a phospholipid bilayer is almost equal for holotoxin and the isolated A-chain [7].

Hybridoma cells producing monoclonal antibodies (mAb) against plant toxins are convenient models for investigating both the toxin pathway in the mammalian cell and the significance of protein epitopes for toxin-mediated cytotoxicity. We developed a panel of mAbs to mistletoe lectin (ML), and present evidence for the role of disulfide bond reduction and subunit dissociation as an obligate step in toxin translocation into the cytosol. The anti-MLA mAb MNA5 does not bind the holotoxin; the interaction of the mAb with the toxin after reducing treatment and protein heat denaturation is characterized. Our cytotoxic experiments with hybridomas reveal that the interchain disulfide bond reduction and dissociation of the subunits is a prerequisite for cytotoxicity.

## 2. Materials and methods

### 2.1. Materials

Cell culture medium RPMI 1640, L-glutamine, kanamycin, and fetal calf serum were from Flow Labs (Woodcock Hill, UK); protein A-Sepharose from LKB-Pharmacia (Uppsala, Sweden); 96-well microtiter plates from Nunclon (Roskilde, Denmark); streptavidin labelled with peroxidase and polyclonal rabbit anti-mouse immunoglobulins from IMTEK (Moscow, Russia); all other reagents were obtained from Sigma (St. Louis, MO, USA).

### 2.2. Protein purification

Mistletoe lectin and its subunits were isolated as previously described [8]. The chimeric toxin consisting of MLA and B subunit of ricin was prepared as previously described [9]. To produce the hybrid-

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**Abbreviations:** DTT, dithiothreitol; IC<sub>50</sub>, toxin concentration causing death of 50% of cells; mAb, monoclonal antibody; ML, mistletoe lectin; MLA, A-chain of ML; MLB, B-chain of ML; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RTB, B-chain of ricin; PBS, phosphate buffered saline; RIP, ribosome-inactivating protein

omas to MLA, spleen cells from BALB/c mice immunized with MLA were hybridized with SP2/0 murine myeloma cells. The hybridomas were tested by ELISA with biotinylated ML and MLA. mAbs from ascitic fluid were purified by affinity chromatography on protein A-Sepharose. Biotinylation was performed with biotinamidocaproate *N*-hydroxysuccinimide ester as recommended by Sigma. Protein purity was estimated with SDS polyacrylamide gel electrophoresis (10%).

### 2.3. Binding and cytotoxic assays

The interaction of mAbs with mistletoe lectin was analyzed in ELISA; the data were read with a Multiscan spectrophotometer (LabSystems, Finland). Mistletoe lectin cytotoxicity was estimated by cell survival in the MTT test [10].  $IC_{50}$  was used to compare the cytotoxic activity of different proteins.

### 2.4. Epitope prediction and peptide synthesis

Epitope prediction was carried out according to [11] using the DSSP program. The mistletoe lectin and ricin amino acid sequences were aligned using the FASTA program and Swiss-Prot data base. Protein fragments each containing eight amino acid residues of MLA were synthesized as a special application of PEPSCAN (peptide scanning approach) [12]. Peptides were synthesized and attached to polyethylene pins by the active ester coupling procedure using 9-fluorenylmethyloxycarbonyl-amino acid-pentafluorophenyl esters in the presence of an equimolar amount of 1-hydroxybenzotriazole according to the manufacturer's instructions (Cambridge Research Biochemicals Co., UK). Interaction of mAbs with the peptides was analyzed by ELISA.

## 3. Results and discussion

After screening, we picked out three clones producing antibodies reacting most effectively with isolated native MLA: MNA4, MNA5 and MNA9.

mAb MNA5, in contrast to the other mAbs, does not interact with native mistletoe lectin, chimeric toxin MLA/RTB (Fig. 1), or MLA absorbed on a microtiter plate. All other mAbs react with both native and absorbed forms of ML. Evidently, MNA5 recognizes a conformational epitope which is hidden by the B-subunit in the native molecule of ML. mAbs MNA4 and MNA9 bind the holotoxin and isolated MLA equally.

To investigate the conditions of MNA5 epitope exposure in ML, the holotoxin was dissociated by reducing the S-S bond with dithiothreitol (DTT). This was verified by sandwich ELISA with mAbs against MLA and mAb TB12 against MLB (data not shown). DTT elicited the MNA5 epitope after dissociation of the subunits. Denaturation of mistletoe lectin by heating to 100°C did not yield an MNA5-reactive epitope. The endocytosed toxin has been shown to be routed to the Golgi apparatus via acidic endosomes. The pH 5.5 treatment of ML in our experiments had no effect on the binding of MNA5 to the holotoxin (data not shown).

For MNA5 epitope prediction in MLA, we used three-dimensional structure analysis of the monomeric and dimeric protein forms [13]. Amino acids that changed their solvent accessibility after A-B heterodimer formation were revealed; these were numbers 18, 35–38, 170–171, 174, 177–178, 191, 210–211, 213, 215, 222, 228–229, 231–232, 234–235, 237–241, 248–252. Then we chose amino acid residues in the regions with maximum mobility and temperature B-factor and not included in the secondary structure elements in the X-ray model [14]. The final choice included the analysis of amino acid sequence variability in homologous proteins; non-conservative domains had maximum preference. As a result, the following possible MNA5 epitopes were proposed: 248–254, 222–229, 210–219 and 174–182. We have investigated the in-

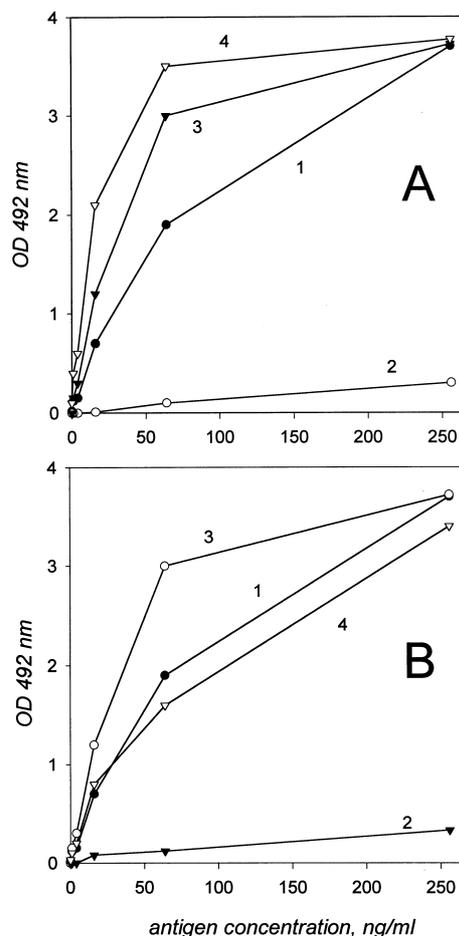


Fig. 1. Interaction of mAbs MNA5 (1, 2) and MNA9 (3, 4) with antigens in sandwich ELISA with biotinylated MNA4. A: MLA (1, 3), ML (2, 4). B: MLA (1, 3), MLA/RTB (2, 4). mAbs at 0.01 mg/ml PBS were adsorbed for 16 h at 4°C on microtiter plates, which were then reacted at 37°C for 60 min with the antigen and 60 min with biotinylated Abs (with washing at each step), and developed with streptavidin-peroxidase.

teraction of the mAbs and of polyclonal mouse serum with a panel of octapeptides of MLA synthesized and linked by their C-terminal carboxyl group to the surface of polyethylene pins (Fig. 2). Polypeptides numbered 1–2 include residues 174–182; nos. 3–6, 196–206; nos. 7–9, 210–219; nos. 10, 222–229; nos. 11–12, 231–239; nos. 13, 248–255. Polyclonal antibodies from the serum of MLA immunized mice whose spleen cells had been taken for fusion with SP2/0 cells to produce hybridomas interacted mainly with two fragments containing residues 231–238 and 232–239 from the MLA sequence (Fig. 2). However, our mAbs did not bind with the surface-attached MLA fragments. This demonstrates again that MNA5 cannot interact with denatured (unfolded) MLA fragments.

The cytotoxic activity of mistletoe lectin and ricin against hybridoma cells producing anti-MLA mAbs is presented in Fig. 3. The sensitivity of MNA4 and MNA5 hybridoma cells to ML is 70 times less than that of SP2/0 cells. The  $IC_{50}$  of chimeric toxin MLA/RTB for MNA5 cells is 40 times higher than for control SP2/0 cells. The sensitivity of SP2/0 and TA75 hybridoma cells against denatured MLA to ML is equal. ELISA shows that no mAb binds ricin and that the hybridomas have equal sensitivity to this toxin.

The sensitivity of hybridoma cells producing mAbs to ML

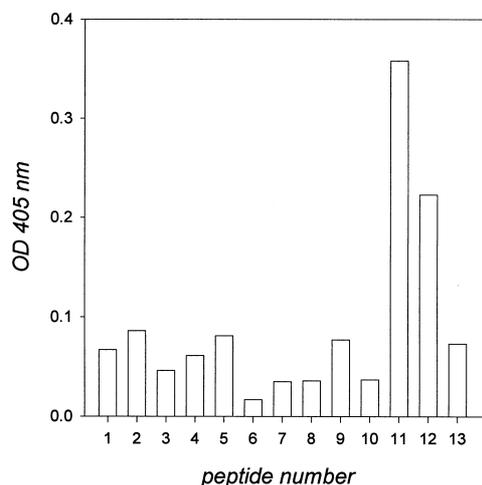


Fig. 2. Interaction of the anti-MLA polyclonal mouse serum with a panel of octapeptides of MLA. Octapeptides of MLA were synthesized and linked to the surface of polyethylene pins. The plate with pins was incubated with the mouse serum diluted 1:100 for 60 min at 37°C, washed and developed with rabbit anti-mouse IgG-peroxidase.

depends on the intracellular interaction of mAb with toxin molecules. Additionally, mAbs in the hybridoma culture media might prevent ML binding to the cells. We excluded this binding by repeated washing of cells with cell culture media without serum. The maximum number of binding sites for ML on specific and control hybridoma cells does not differ (data not shown). Papain treatment of the cells to eliminate external mAb molecules does not change the sensitivity to the toxin [15]. Thus, hybridoma cell resistance is due to intracellular interaction of toxins with newly synthesized antibody molecules.

The resistance of MNA5 hybridoma cells to ML shows that mAb molecules bind toxin molecules after reduction of the disulfide bond between the A- and B-chains of the toxin and its dissociation. The reduction could take place in the

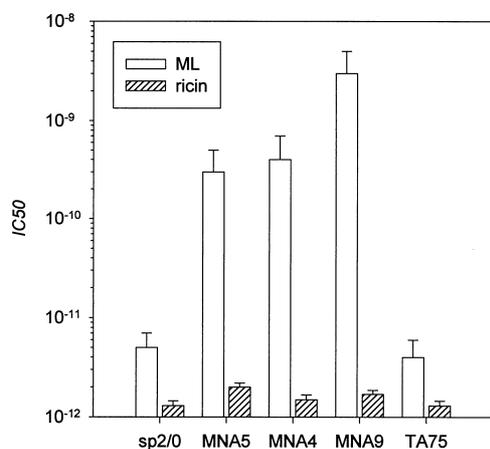


Fig. 3. Cytotoxic activity of mistletoe lectin and ricin in hybridoma cells synthesizing mAbs against MLA. The cells were incubated ( $4 \times 10^4$  per well) in 0.15 ml of medium with various toxin concentrations at 4°C for 1 h. After washing to remove non-bound protein and incubation in a CO<sub>2</sub> incubator at 37°C for 36 h, 0.015 ml of MTT solution (5 mg/ml in PBS) was added into each well for 3 h at 37°C. The cell pellet was dissolved by adding 0.1 ml isopropanol into each well. The optical density was determined at 600 nm.

ER containing a redox complex, i.e. reduced glutathione and protein disulfide isomerase [1]. After the A-chain has been translocated from the ER lumen, it may utilize, in the reverse direction, the ER membrane translocation apparatus normally involved in the co-translational translocation of secretory proteins across the mammalian ER membrane, the Sec61p complex [16]. No direct evidence for the reverse translocation step has yet been obtained. Conceivably, the A-chain export to the cytosol could utilize an alternative translocator. However, cells defective in their TAP1 and TAP2 proteins, normally responsible for transporting peptides across the ER membrane for presentation by the MHC class I molecules, remain completely sensitive to ricin, indicating that the A-chain does not use the peptide translocator in the reverse direction [1,4].

Both hydrophobic and hydrophilic amino acids take part in the contact between the A- and B-subunits of ML [17]. These are mainly the C-terminal residues not exposed in the holotoxin. The important role of these residues in ricin translocation was shown in cytotoxicity experiments with recombinant ricin A-chain containing Ala instead of Pro-250. This substitution dramatically decreased the cytotoxic activity of re-associated holotoxin [18]. Comparative amino acid analysis of ricin and ML shows that MLA has residues with a high hydrophobic index in the interchain region. The main antigenic epitopes of ML include these residues, as shown by our analysis.

The comparison of the amino acid sequences of ricin and ML with other RIP II toxins, namely nigrin b and ebulin [19,20], which have low cytotoxic activity, shows pronounced differences between the C-ends of their A-chains. No similar region is also found in gelonin, a representative of type I RIP. Receptor-mediated conjugates of gelonin have less cytotoxic activity than ricin or ML A-chain immunotoxins [21]. Thus, our study of ML activity on MNA5 hybridoma cells revealed that the disulfide bond is reduced and that the chains dissociate before the MLA is translocated into the cytosol. The intramolecular domain of MLA exposed upon subunit dissociation plays a crucial role in transmembrane penetration.

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